PRODUCTION OF OLIGOSACCHARIDES IN TRANSGENIC PLANTS

The present invention relates to a method for producing oligosaccharides, to the oligosaccharides produced in this manner, to transgenic plants and plant cells capable of producing oligosaccharides and to the applications of the oligosaccharides obtained in this manner.

In the food industry a growing trend toward "light" and ~~low-calory~~ can be observed. The use ~~herein~~ of too much fat and/or sugar in products is avoided. To nevertheless be able to provide food products with a sweet taste, an increasing number of sugar substitutes are becoming commercially available. Aspartame is a known example thereof. Aspartame, however, has poor organoleptic properties.

Another type of sugar substitute is formed by oligosaccharides. Oligosaccharides are molecules which consist of two or more monosaccharides such as fructose and/or glucose. The monosaccharides in ~~the said~~ oligosaccharides are linked to each other either by ß-(2-1)- or by ß-(2-6) bonds. The number of monosaccharides in an oligosaccharide is indicated by means of the DP-value ("Degree of Polymerisation"). A DP-value of 3 means that the oligosaccharide is composed of three monosaccharides. Oligofructoses are oligosaccharides consisting entirely of fructose units. When an oligosaccharide also comprises one or more glucose units these will be linked by means of an ß-(1-2) bond to a fructose unit. The composition of oligosaccharides is also designated with the formula G_mF_n, wherein G represents glucose and F fructose and wherein m equals 0 or 1 and n is an integer larger than or equal to 0. Particularly suitable oligosaccharides are those wherein m equals 1 and n is 2 to 8, preferably 2 or 3.

Oligosaccharides can hardly be hydrolysed, if at all, in the human stomach and small intestine. It is known of oligofructose that the digestive enzymes of the human have no effect on the ß(2-1) and ß(2-6) bond in the molecule. They therefore pass through the stomach and the small intestine without being degraded and absorbed into the body. The oligosaccharides do not however leave the body but are me-

kilojoule per gram. In addition, free sugars cause dental decay (caries).

Conversely, oligosaccharides with too high a chain length have too little sweetening power, which causes the average sweetening power of the mixture to fall.

In contrast to some other sweeteners such as for example Aspartame, oligosaccharides have good organoleptic properties.

It is the object of the present invention to provide an alternative production route for oligosaccharides with which the above stated drawbacks are avoided.

To this end the invention provides a method for producing oligosaccharides, comprising the steps of:

a) selecting a gene which codes for an enzyme capable of converting sucrose into an oligosaccharide;

b) linking the gene to suitable transcription-initiation and transcription-termination signals in order to provide an expression construct;

c) transforming a suitable plant cell with the expression construct;

d) regenerating a transgenic plant from the transformed plant cell;

e) culturing the transgenic plant under conditions enabling the expression and activity of the enzyme; and

f) isolating the oligosaccharides from the transgenic plant.

The invention therefore provides a method with which by means of transgenic plants or plant cells an oligosaccharide or a mixture of oligosaccharides can be produced which have more desirable properties compared with the oligosaccharides prepared by known industrial processes.

The particular advantage of the method according to the invention is that the chain length distribution is narrower, whereby no or few free sugars occur in the end product. The consequence hereof is a lower cariogenicity and the desired lower energy value. There also occur fewer oligosaccharides with a chain length of more than 5. The advantage hereof is that the oligosaccharides produced according to the inventi-

on have a higher specific sweetening capacity. It is the case that the sweetening capacity depends on the "average chain length". The higher the average chain length of a mixture, the lower the sweetening capacity. The advantage of a high specific sweetening capacity is that extra sweeteners hardly have to added in processing of the product.

A similar consideration applies in respect of solubility. It is also the case here that when the average chain length increases the solubility deceases. The mixtures according to the invention therefore have a higher solubility than the mixtures obtained by means of enzymatic synthesis or enzymatic hydrolysis. In addition, production costs are considerably reduced.

There are indications that short chains can be absorbed better in the bacteria body of Bifidus than long ones. The oligosaccharide mixtures produced by means of the method according to the invention will therefore have a higher bifidogenic effect.

In order to select a gene which codes for an enzyme capable of converting sucrose into an oligosaccharide it is possible to search in any possible organism which contains fructosyltransferase activity, for instance micro-organisms such as bacteria, or plants. It is known of many micro-organisms that they contain fructosyltransferases which are capable of producing fructans from sucrose. These enzymes transfer fructose units from sucrose to a fructan acceptor molecule. Microbial fructosyltransferases normally produce fructans with a high DP. The use of a number of fructosyltransferases to manufacture transgenic plants for the production of such polysaccharides is already described in the literature. It is thus known that by incorporating the SacB-gene of Bacillus subtilis in plants the fructan pattern of these plants can be modified (WO 89/12386). This still relates however to the production of high-molecular polysaccharides.

Another gene which is known to code for a fructosyltransferase which can convert sucrose into high-molecular fructans is the ftf gene of Streptococcus mutans. According

to the present invention It has now been found surprisingly that in addition to high-molecular fructans this fructosyltransferase also produces significant quantities of oligosaccharides in the trisaccharide class (1-kestose). Mutants have also been found which only accumulate trisaccharides and not polysaccharides.

Further known are mutants of the SacB gene of Bacillus subtilis which likewise produce mainly trisaccharides.

A large number of other micro-organisms is likewise capable of fructosyltransferase production. These comprise, but are not limited, to endospore-forming, rod bacteria and cocci (for example Bacillus), gram-positive cocci (for instance Streptococcus), gram-negative aerobic rod bacteria and cocci (for instance Pseudomonas, Xanthomonas, Azotobacter) gram-negative facultative anaerobic rod bacteria (for instance Erwinia, Zymomonas), actinomycetes (for instance Actinomyces, Rothia) and cyanobacteria (for instance Tolypothrix tenuis).

The genes which code for these fructosyltransferases can optionally be modified by targeted or random mutagenesis techniques in order to provide enzymes possessing the desired oligosaccharide-synthesizing enzymatic properties.

Bacterial fructosyltransferases have a relatively low KM for sucrose, approximately 20 mM. The sucrose concentrations in most plants is considerably higher and these enzymes will therefore also be active in plants. An important property of bacterial fructosyltransferase is their activity at low temperatures to 0°C. Plants often come into contact with these temperatures but the bacterial enzymes will still be active even under these conditions.

Fructosyltransferases can also be of vegetable origin. In plants the biosynthesis and degradation of fructan only occur in a limited number of species. Examples are the Asteraceae, Liliaceae and Poaceae families. Starting from the known vegetable fructosyltransferases, the genes suitable for the present invention can be isolated or manufactured either by targeted or random mutagenesis or by selection of already naturally occurring mutants.

RNA4 translation enhancer signal, which must be present in the transcribed 5' non-translated region.

For correct termination of transcription a terminator sequence can be added to the constructs. An example of such a sequence is the nopaline synthase gene termination sequence.

The choice of expression signals suitable for a specific situation lies of course within the reach of the average skilled person without further inventive work having to be performed for this purpose.

Sucrose, the substrate for the fructosyltransferases, is a carbohydrate present at many different locations. It is synthesized in the cytoplasm and significant quantities can also be found in cytosol, vacuole and the extracellular space (the apoplast) or other possible locations.

Since biochemical processes in plant cells are likewise often limited to a single or a number of cellular compartments, it is desirable to cause the accumulation of the products of the newly introduced genes to take place in a specific compartment. For this purpose targeting sequences which are specific to cellular compartments can be present in the expression construct close to the coding part of the fructosyltransferase genes which are expressed in the transgenic plants. Specific amino acid regions for the targeting to the different cellular locations have already been identified and analysed. These DNA-sequences can be linked to the fructosyltransferase genes such that the enzymatic activity is directed to a desired compartment of the cell or the plant.

In a preferred embodiment of the invention the expression construct therefore also comprises a targeting sequence for directing the fructosyltransferase activity to one or more specific plant cell compartments. Examples of targeting sequences are the signal sequence and vacuolar targeting sequence of the carboxypeptidase Y (cpy) gene, that of patatine from the potato or that of sporamine from the sweet potato, or the signal sequence and apoplastic targeting sequence of the pathogenesis-related protein S-gene (pr-s).

These are examples, and the skilled person will himself be capable of selecting other targeting sequences.

The expression construct can in principe be modified such that targeting takes place to any random cell compartment, such as the vacuole, plastides, cell wall, cytoplasm etc.

It is often advantageous for the plant to control not only the location but also the time of expression of the introduced genes. It is for instance normally desired to limit the expression of the newly introduced enzymatic activities to specific parts of the plant, for instance harvestable organs such as tubers, fruits or seeds. It is moreover often desired to initiate expression in these organs at a particular stage of development. This is certainly the case when the expression of the introduced genes interferes with normal development of such organs.

The oligosaccharides according to the invention can be used as substitute for sugar, glucose syrup and isoglucose in "light" versions of different food products. Examples of food products are confectionery, biscuits, cakes, dairy products, baby food, ice cream and other desserts, chocolate and the like. The stimulation of Bifidobacteria is also important for the health of animals. The oligosaccharides according to the invention can therefore also be applied in for instance animal feed.

The present invention will be further elucidated on the basis of the examples hereinbelow, which are only given by way of illustration of the invention and are not intended to limit it in any way. Reference is made in the examples to the annexed figures which show the following:

Figure 1 shows the oligosaccharide-producing activity of wildtype and modified forms of the Streptococcus mutans fructosyltransferase (ftf) which is incubated with sucrose and analysed on TLC as described by Cairns, A.J. and Pollock, C.J., New Phytol. 109, 399-405 (1988). Samples of cultures which were derived from colonies and purified proteins were incubated overnight with 200 mM sucrose in 50 mM sodium phosphate buffer with 1% Triton X-100 at 37°C.

Lane 1 shows the reaction products of an S. mutans culture; lane 2 shows the activity of the purified enzyme from S. mutans; lane 3 shows the activity of an E. coli strain harbouring the plasmid pTS102; lane 4 shows the activity of an E. coli strain harbouring plasmid pTD2; lane 5 shows the activity of an E. coli cell which is transformed with the mature S. mutans fructosyltransferase gene under the regulation of an E. coli promotor. The oligosaccharide standards used are in lane A an extract of an Allium cepa bulb, and in lane H an extract of a Helianthus tuberosus tuber. In the figure F represents fructose, G glucose, S sucrose (disaccharide), N neokestose (F2-6G1-2F, trisaccharide), I represents 1-kestose (G1-2F1-2F, trisaccharide), K represents kestose (G1-2F6-2F, trisaccharide). Higher oligosaccharides (DP = 4-9) are likewise indicated.

Figure 2 shows the TLC-analysis of transgenic tobacco plants (KZ) which express the fructosyltransferase gene of S. mutans. Oligosaccharides accumulate in these plants. Lane H shows as control an extract of a Helianthus tuberosus tuber.

Figure 3 shows the SDS-PAGE gel of purified SST from onion seed. A single band was visible in the SST sample on this gel stained by means of silver-staining. M represents molecular weight markers wherein their size is indicated in kilodaltons (kD).

Figure 4 shows the reaction products of purified SST from onion seed which is incubated with sucrose (lanes 4 and 5: O-in vitro). Only trisaccharides are formed. Lane 1 shows the extract of tulip stalks (T), lane 2 the extract of Helianthus tuberosus tubers (H), lane 3 shows the extract of an Allium cepa bulb (O). M represents monosaccharide, S sucrose (disaccharide), N neokestose (F2-6G1-2F, trisaccharide), I represents 1-kestose (G1-2F1-2F, trisaccharide). Higher oligosaccharides (DP4-5) are likewise indicated. The products were analysed on TLC as described for figure 1.

Figure 5 shows the separation of 2 isoforms of the sucrose-fructan 6-fructosyltransferase (6-SFT) from barley after the second anion exchange chromatography step on a

Resource Q column in a purification procedure. Figure 5A shows the protein elution profile (A280) and the fructosyltransferase activity of the fractions obtained after chromatography after incubation with 0.2M sucrose in 25 mM methylpiperazine (HCl) buffer (pH 5.75). De chromatograms (Fig. 5B) were obtained by pulsed amperometric detection after anion exchange HPLC separation on a CarboPack-PA100 column. The reaction products were obtained after incubation of pool I and pool III with sucrose alone, or sucrose and isokestose. The carbohydrates were identified by their retention times and trehalose was used as internal standard.

Open circles in fig. 5A represent fructosyltransferase activity, which is indicated as the sum of formed kestose, bifurcose, isokestine and kestine. In fig. 5B p corresponds with a non-identified product resulting from isokestose contaminants, and c with a contamination of the isokestose substrate.

Figure 6A shows a graph of the enzymatic activity of a pool of fractions of 6-SFT (referred to as pool II; see figure 5) after isoelectric focussing under non-denaturing conditions. Closed triangles indicate beta-fructosidase activity measured as released fructose, while open circles indicate the fructosyltransferase activity measured as formed kestose. Figure 6B is an SDS-PAGE gel after two-dimensional analysis of pool II after the second anion exchange chromatography. The two 6-SFT isoforms are shown herein. Both isoforms are found to consist of two subunits of respectively 23 kDa and 49 kDa. Figure 6C is the two-dimensional gel electrophoresis of the IEF-markers phycocyanin (pI 4.6), beta-lactoglobulin (pI 5.1) and bovine carbonic anhydrase (pI 6.0).

Figure 7 is a schematic view of the strategy used to obtain the cDNA clone which codes for 6-SFT from barley.

Figure 8 shows the cDNA-sequence and the amino acid sequence of 6-SFT from barley derived therefrom.

Figure 9 is an overview of the derived amino acid sequence of 6-SFT from barley, different invertases (beta-fructosidases), levanases and levansucrases. The overview

was produced with the Pileup program of the GCG sequence analysis software package. The following abbreviations were used:

H.v. 6-SFT = sucrose-fructan 6-fructosyltransferase from barley.

V.r. Inv = soluble acid invertase from green soya bean (mungbean; Arai et al., Plant Cell Physiol. 33, 245-252 (1992));

D.c. Inv = soluble acid invertase of carrot (Unger et al., Plant Physiol. 104, 1351-1357 (1994));

L.e. Inv = soluble acid invertase of tomato (Elliott et al., Plant Mol. Biol. 21, 515-524 (1993));

D.c. cw Inv = cell wall invertase of carrot (Sturm and Crispeels, Plant Cell 2, 1107-1119 (1990));

A.s. Inv = partial invertase sequence of oats (Wu et al., J. Plant Physiol. 142, 179-183 (1993));

E.c. Inv = invertase (rafD) of Escherichia coli (Aslandis et al., J. Bacteriol. 171, 6753-6763 (1989));

S.m. Scrb = invertase of Streptococcus mutans (Sato and Kuramitsu, Infect. Immun. 56, 1956-1960 (1989));

B.p. LelA = levanase from Bacillus polymyxa (Bezzate et al., non-published reference EMBO data base);

B.s. SacC = levanase of Bacillus subtilis (Martin et al., Mol. Gen. Genet. 208, 177-184 (1987));

K.m. Inu = inulinase of Kluiveromyces marxianus (Laloux et al., FEBS Lett. 289, 64-68 (1991));

S.c Inv1 = invertase 1 of baking yeast (Hohmann and Gozalbo, Mol. Gen. Genet. 211, 446-454 (1988));

S.o. inv = invertase of Schwanniomyces occidentalis (Klein et al., Curr. Genet. 16, 145-152 (1989));

A.n.Inv = invertase of Aspergillus niger (Boddy et al., Curr. Genet. 24, 60-66 (1993));

B.a. SacB = levansucrase of Bacillus amyloquefaciens (Tang et al., Gene 96, 89-93 (1990));

B.s. SacB = levansucrase of Bacillus subtilis (Steinmetz et al., Mol. Gen. Genet. 200, 220-228 (1985));

S.m. SacB = levansucrase of Streptococcus mutans (Shiroza and Kuramitsu, J. Bacteriol. 170, 810-816 (1988));

Z.m. LevU = levansucrase of Zymomonas mobilis (Song et al., non-published reference in EMBO database).

Figure 10 is a dendrogram of 6-SFT from barley with different invertases (beta-fructosidases), levanases and levansucrases, based on derived amino acid sequences. The dendrogram was generated with the sequences described in figure 9 making use of the Pileup program of the GCG sequence analysis software package.

Figure 11 shows the functional expression of barley 6-SFT in Nicotiana plumbaginifolia protoplasts. Error bars indicate the average standard deviation. The 6-SFT cDNA was expressed for 27 hours in protoplasts. Samples were taken a number of times and the fructosyltransferase activity was determined in protoplast extracts by incubation with sucrose (Fig. 11A) or sucrose and isokestose (Fig. 11B). Open circles show the enzyme activity of extracts of protoplasts which were transformed with the 6-SFT gene construct. Open squares show the activity of extracts of protoplasts transformed with the vector without the 6-SFT cDNA.

Figure 12 is a native IEF-gel of a purified enzyme extract of fructan-fructan fructosyltransferase (FFT) from Helianthus tuberosus L.. After Coomassie Blue staining there can be seen in addition to the two most important isoforms of the FFT (T1 (pI 4.45) and T2 (pI 4.75)) a band with a pI of approximately 5.5, which probably corresponds with denatured FFT.

Figures 13 and 14 are HPLC-diagrams of tryptic digests of the FFT isoforms T1 (Fig. 13) and T2 (Fig. 14).

EXAMPLE 1

Selection of a gene.

1. Naturally occurring genes.

A large number of microbes was screened for their capacity to produce oligosaccharides from sucrose. For this purpose bacteria cultures were grown overnight in a liquid nutrient. The oligosaccharide-producing activity was determined by incubating a sample of the culture with 200 mM sucrose in the presence of 0.1% Triton X-100. The reaction products were separated by means of TLC and made visible using a fructose-specific reagent (Cairns, A.J. and Pollock, C.J., New Phytol. 109, 399-405 (1988)). It was found as a result of this screening that Streptococcus mutans is an effective producer of oligosaccharides (see figure 1). The oligosaccharide-producing enzymatic activity was purified from the Streptococcus mutans culture by means of DEAE-ion exchange chromatography and gel permeation chromatography. It was found herefrom that the enzymatic activity was caused by the product of the ftf gene previously described by Shiroza and Kuramitsu, (J. Bacteriol, 170, 810-816 (1988)).

The fructosyltransferase (ftf) gene from plasmid pTS102 (Shiroza and Kuramitsu supra) was subsequently cloned as an EcoRV-BglII fragment in the multiple cloning site of pEMBL9 (Dente et al., Nucl. Acids Res. 11, 1645-1655 (1983) and expressed from the lacZ promotor present in this plasmid. E. coli was then transformed herewith. The bacteria was hereby made capable of producing oligosaccharides.

The production of oligosaccharides was demonstrated by means of the screening method already mentioned above. Non-transformed E. coli does not produce any oligosaccharides from sucrose.

2. Mutated genes.

By means of mutagenesis it is possible to adapt the oligosaccharide-producing activity of the enzyme as required. Mutations in the gene can be brought about for instance in the following manner.

For mutagenesis of the ftf gene of Streptococcus mutans the plasmid pTS102 was integrated into the genome of Synechococcus sp. PCC 7942 (R2-PIM9) by means of the genomic integration system (Van der Plas et al., Gene 95, 39-48 (1990)), which resulted in strain R2-PTS. This cyanobacteria R2-PTS strain expresses the fructosyltransferase gene. The R2-PTS strain is sucrose-sensitive due to polymer accumulation in the periplasm. An R2-PTS culture was mutated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which induces point mutations (T → C and G → A mutations) Mutants with a changed fructosyltransferase activity were selected. The culture mutated by means of MNNG was plated on sucrose-containing medium and a total of 400 sucrose-resistant colonies were tested for a changed fructosyltransferase activity.

Derived from these colonies were R2-PTS cultures which were concentrated by means of centrifugation. The thus obtained pellets were resuspended in 50 mM sodium phosphate buffer with 1% Triton X-100, 200 mM sucrose and incubated overnight at 37°C. The reaction products were analysed by means of TLC-analysis (Cairns and Pollock supra). The TLC was developed three times in 85:15 acetone:water and subsequently treated with atomized urea as described by Wise et al., Analytical Chemistry 27, 33-36 (1955). This method preferably stains fructose and fructose-containing polymers.

Of the mutants substantially producing trisaccharides one was chosen for in vitro demonstration of the enzymatic oligosaccharide-forming activity of the mutated ftf gene in the above described manner.

According to the invention other mutagenesis methods (site-directed or random) and genes which code for fructosyltransferases from other organisms can likewise be used to select a gene for a mutant oligosaccharide-producing protein.

EXAMPLE 2

Expression of the ftf gene in plants.

A. Construction of 35S-ftf-NOS in a plant transformation vector

The plasmid pMOG18 which contains a plant-specific 35S promotor with an enhancer duplication and sequences which stimulate the translations of mRNA is described by Symons et al. (Bio/Technology 8, 217-221 (1990)). It contains the 35S-promotor-uidA-gene-NOS-terminator construct. A pBluescript II SK-plasmid from Stratagene (San Diego, CA, U.S.A.), from which the internal BamHI-site was removed by digestion with BamHI and filling in the sticky ends with Klenow and ligating once again, was used for further cloning. The 35S-uidA-NOS-fragment was obtained by digestion with EcoRI and HindIII of pMOG18 and in this BamHI-pBluescript was cloned in the corresponding EcoRI/HindIII site, resulting in plasmid pPA2. Plasmid pPA2 was digested with NcoI and BamHI and the vector-containing fragment was isolated.

The fructosyltransferase gene ftf was cloned from the plasmid pTS102 (see above) as an EcoRV/BglII fragment in the multiple cloning site of pEMBL9. The compatible SmaI- and BamHI locations were used for this purpose. This resulted in the plasmid pTA12.

In order to obtain an NcoI location close to the mature processing site of the ftf gene (nucleotide position 783) (J. Bacteriol. 170, 810-816 (1988)), site-directed mutagenesis was performed as described by Kramer et al. (Nucleic Acids Res. 12, 9441-9456 (1984)) with the following oligonucleotide: 5'-GGCTCTCTTCTGTTCCATGGCAGATGAAGC-3'. Resulting herefrom was plasmid pTD2. At amino acid position +1 (nucleotide position 783) relative to the mature processing site a glutamine was hereby changed into a methionine. The NcoI/PstI fragment in which the sequence coding for the mature fructosyltransferase is present was used for further cloning. From this plasmid the ftf gene was isolated as an NcoI/PstI fragment and this fragment was ligated in the pPA2

vector-containing fragment described above. This results in plasmid pTX. pTX contains the 35S-ftf-NOS-fragment in which ftf shows the mature fructosyltransferase gene without its signal sequence region. pTX was digested with XbaI and HindIII, the fragment containing the complete construct (35S-ftf-NOS) was cloned in the XbaI/HindIII restriction site of pMOG23 (Symons et al., supra) a derivative of the binary plant vector pBIN19 (Bevan, Nucl. Acids. Res. 12, 8711-8721). This resulted in plasmid pTZ.

B. Manufacture and analysis of transgenic plants which express the mature ftf gene

The pTZ-plasmid was conjugated in Agrobacterium tumefaciens LB4404 (Hoekema et al., Nature 303, 179-180 (1983)) in a three-point crossbreeding making use of the helper plasmid pRK2013 (Lam, Plasmid 13, 200-204 (1985)). The construct was introduced into Nicotiana tabacum var. Petit Havanna (SR1) using the leaf disc transformation method (Horsch et al., Science 227, 1229-1232 (1985)). The regenerated plants were called KP-plants and were selected for kanamycine resistance and cultured on MS medium (Murashige and Skoog, Physiol. Plant. 15, 473-497 (1962)). Thereafter the plants were grown on soil in the greenhouse and analysed.

The leaf material was cut off and ground in an eppendorf tube. After centrifugation (2 minutes at 16,000 rpm) 1 µl supernatant was analysed on TLC as described in example 1.

Oligosaccharides were never found in wildtype plants or in plants which were transformed with non-related constructs. The screening of the transformants demonstrated oligosaccharide-accumulating plants using this method (see figure 2). The expression levels varied between individual plants which were transformed with the same construct. This is a normal phenomenon in transformation experiments in plants. The variation of the expression levels depends substantially on the integration position in the genome (position effect).

EXAMPLE 3

Oligosaccharide-producing enzyme (SST) from the onion

In addition to the above used fructosyltransferase genes originating from micro-organisms, such enzymes are also produced by plants. In this example the SST gene from onion seed is used.

The SST protein from onion seed was purified by chromatographic procedures making use of the following protocol. The seed was incubated at 22°C between moist cloths for 2 to 3 days and homogenised in 50 mM phosphate-citrate buffer with a pH of 5.7. The starch and debris were centrifuged off at about 10,000 g for 10 minutes. Ammonium sulphate was added to the supernatant to 20% and the precipitate collected by centrifugation. The concentration of ammonium sulphate in the supernatant was increased to 80% and the precipitate collected and dissolved in 20 mM NaAc pH 4.6. The solution was dialysed overnight with three buffer changes (20 mM NaAc) and the solution clarified by centrifugation. The supernatant was placed on an FPLC monoS-column and eluated in 20 mM NaAc pH 4.6 with a 0-0.5 M NaCl gradient. After dialysis overnight against 10 mM NaAc pH 5.6 the solution was placed onto a raffinose-epoxy sepharose column (Pharmacia), which was equilibrated in 10 mM NaAc pH 5.6. Elution took place with a linear gradient consisting of 10 mM NaAc pH 5.6 (buffer A) and 10 mM phosphate-citrate buffer, pH 7.0, plus 0.5 M NaCl-buffer (buffer B). The active fractions were dialysed overnight against 20 mM phosphate-citrate - buffer, Ph 7.0, and placed on a monoQ FPLC-column in 20 mM phosphate-citrate buffer, pH 7.0. The column was eluated with a gradient of 0-0.5 M NaCl. For a final purification the protein was placed onto a Sepharose 6-column and eluated with 50 mM phosphate buffer, pH 6.5, 1% Triton X-100. The silver staining of an SDS-PAGE gel of purified SST from onion seed revealed only one band with a molecular weight of approximately 68,000 d (see figure 3).

When this purified SST was incubated with sucrose only 1-kestose was produced. No significant invertase activity was observed (see figure 4).

The amino acid sequence of the purified protein was determined on the basis of peptides obtained by gradual breakdown. On the basis of this information PCR-probes were designed with which the gene coding for the SST of onion seed was isolated. In the same manner as described in examples 1 and 2 it was hereby demonstrated both *in vitro* and *in vivo* that the gene codes for an enzyme capable of producing oligosaccharides.

EXAMPLE 4

Applicability with other plant species

In order to illustrate the general applicability of the technology the *ftf* construct described in example 2 was introduced into different crops. The potato was thus transformed according to the method described in Visser, Plant Tissue Culture Manual B5, 1-9, Kluwer Academic Publishers, 1991. The resulting transgenic plants accumulated oligosaccharides in each tested organ. The same construct was also introduced into the beet (*Beta vulgaris* L.) which was transformed as described by D'Halluin et al., Biotechnology 10, 309-314 (1992). The resulting transgenic beet plants accumulated significant quantities of oligosaccharides in for instance their leaves and roots. The same constructs were introduced into *Brassica napus* L. which was transformed as according to Block et al., Plant Physiol. 91, 694-701 (1989). The resulting transgenic plants accumulated significant levels of oligosaccharides in for instance their leaves and storage organs. It is of course not essential that the plants are transformed in the manner indicated. Other methods within the reach of the skilled person can also be used.

Examples of other plant species which can be modified comprise, but are not limited to, maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), soya bean (*Glycin max* L.), pea (*Pisum*

sativum L.), bean (Phaseolus vulgaris L.), chicory (Cichorium intybus L.), sugar cane (Saccharum officinarum L.), sweet potato (Dioscorea esculenta L.), cassava (Manihot esculenta L.) and grasses (for instance Lolium spp., Poa spp. and Festuca spp.).

Plants with natural or induced modified carbohydrate separation patterns can be preferred target plants for the introduction of oligosaccharide-synthesizing genes. Such plants comprise, but are not limited to, natural mutants in starch and sucrose metabolism, and plants in which the starch and sucrose metabolism are modified by means of molecular and genetic techniques, as for instance described in Sonnewald and Willmitzer, Plant Physiology 99, 1267-1270, (1992).

EXAMPLE 5

Sucrose-fructan 6-fructosyltransferase (6-SFT) from barley

1. Introduction

Sucrose-fructan 6-fructosyltransferase (6-SFT) is a key enzyme for the biosynthesis of branched fructans (also called graminans) which are typical for grasses. The enzyme forms kestose from sucrose and bifurcose from sucrose and isokestose. In this example the purification of a 6-SFT from barley (Hordeum vulgare L.) is described, in addition to the cloning of the full cDNA and confirmation of the functionality.

2. Purification of sucrose-fructan 6-fructosyltransferase.

Primary leaves of eight to ten day-old barley plants (Hordeum vulgare L. cv Express) were cut off and exposed to light continuously for 48 hours to induce the accumulation of fructans and the enzymes of the fructan biosynthesis, as described by Simmen et al. Plant Physiol. 101, 459-468 (1993). The leaves were subsequently frozen in liquid nitrogen and stored at -70 °C until they were used.

An enzyme preparation was prepared by grinding induced primary leaves (700 g fresh weight) to a fine powder in liquid nitrogen and subsequently suspending them in extrac-

tion buffer (25 mM methylpiperazine, adjusted to pH 5.75 with HCl, with 1 mM DTT, 1mM benzamidine, 1mM EDTA, 0.1 mM PMSF and 0.5% PVP). 2 ml per g fresh weight hereof was used. After defrosting, the extract was kept at 4°C and adjusted to pH 4.75 by adding 0.1 M HCl in drops while stirring. Three hours later the extract was centrifuged for 30 minutes at 17,000 g. The resulting supernatant was dialysed overnight at 4°C against dialysis buffer (10 mM methylpiperazine (HCl) buffer (pH 5.75), with 1 mM DTT, 1 mM benzamidine, 1 mM EDTA and 0.1 mM PMSF).

The enzyme solution was purified by means of affinity chromatography on Blue Sepharose. For this purpose the enzyme solution was filtered through a 0.45 micrometer Millipore filter and loaded at a flow speed of 2 ml per minute on a column (26x120mm) of Blue Sepharose-6-fast flow (Pharmacia, Uppsala, Sweden), which had previously been equilibrated with the above described dialysis buffer. In order to remove proteins without affinity for the dye the column was washed with three bed volumes of the dialysis buffer. Bound proteins were eluated at a flow speed of 3 ml per minute (5 ml fractions), first with 0.2 M NaCl in 10 mM methylpiperazine (HCl) buffer (pH 5.75) for 30 minutes, followed by a linear gradient of 0.2 M to 0.5 M NaCl in the same buffer within 90 minutes.

All fractions which contained 6-SFT activity were pooled, dialysed overnight at 4°C against dialysis buffer and then concentrated to one third of the starting volume by covering the dialysis bag with polyethylene glycol 40,000 and incubating it for 4 hours at 4°C.

For a first anion exchange chromatography step the 6-SFT fraction was filtered and loaded at a flow speed of 3 ml per minute on a 6 ml resource Q column (Pharmacia), which had been equilibrated earlier with dialysis buffer. After the column was washed with 10 mM methylpiperazine (HCl) buffer (pH 5.75), the bound protein was eluated with a linear gradient of 0 to 0.15 M NaCl in the same buffer within 8 minutes at a flow speed of 15 ml per minute. Fractions of 1 ml were collected. The fractions which contained

6-SFT were pooled and supplemented with ammonium sulphate to a final concentration of 2 M.

The 6-SFT pool was subsequently subjected to hydrophobic interaction chromatography. For this purpose the pool was loaded at a flow speed of 0.5 ml per minute onto an alkylsuperose-column HR5/5 (Pharmacia) which had been equilibrated earlier with 50 mM citric acid-Na_2HPO_4 buffer (pH 5.0) with 2 M ammonium sulphate. The bound proteins were eluated within 60 minutes at a flow speed of 0.5 ml per minute with a linear gradient of 2 to 0 M ammonium sulphate in 50 mM citric acid-Na_2HPO_4 buffer (pH 5.0). Fractions of 0.5 ml were collected and the fractions which contained 6-SFT activity were pooled.

The pooled fractions were subjected to gel filtration chromatography and prior thereto first concentrated to a total volume of 190 microlitres in microconcentrator centrifuge tubes (Centricon-30, Amicon-Grace, Beverly, CT). The concentrate was placed on a Superdex 75 HR 10/30 gel filtration column (Pharmacia), which was equilibrated with 100 mM citric acid-Na_2HPO_4 buffer (pH 5.75) with 0.2 M NaCl, and eluated with the same buffer at a flow speed of 0.4 ml per minute. Fractions of 0.2 ml were collected and the fractions containing 6-SFT activity were pooled and desalted by 5 successive concentrations and dilution steps in Centricon-30 microconcentrator centrifuge tubes with 10 mM methylpiperazine (HCl) buffer (pH 5.75).

For a second anion exchange chromatography step the desalted sample was loaded onto a 6 ml Resource Q column (Pharmacia). The conditions and buffers were the same as for the first anion exchange chromatography step but the fraction size was reduced to 0.5 ml. The fractions which contained 6-SFT activity were combined in pool I, II and III (fig. 5A).

During purification the enzymatic activity of the fractions was determined after the different purifying steps. For this purpose portions of 50-100 µl of the enzyme preparations were desalted by guiding them over Biogel P-10 columns (8x300 mm) by centrifugation at 350 g for 5 minutes

(Simmen et al., supra). Desalted enzyme preparations were incubated with 0.2 M sucrose in 50 mM citric acid-Na_2HPO_4 buffer (pH 5.75) to identify fractions containing 6-SFT activity during the purification. The final enzyme preparations (pool I and III) were incubated with 0.1 M sucrose alone or in combination with 0.1 M isokestose in 25 mM methylpiperazine (HCl) buffer (pH 5.75). Unless otherwise indicated, the enzyme activity assays were performed for three hours at 27°C. The reaction was stopped by heating the samples for 3 minutes at 95°C. The samples were centrifuged for 5 minutes at 13,000 g, supplemented with trehalose (internal standard) to a final concentration of 0.1 µg/µl, and stored at -20°C until the analysis.

Neutral carbohydrates were analysed by means of anion exchange chromatography on a CarboPac PA-100 column (Dionex, Sunnyvale, USA) with a Dionex DX-300 gradient chromatography system coupled to pulsed amperometric detection (Simmen et. al., supra). Prior to analysis by means of anion exchange chromatography, enzyme activities freeing glucose from sucrose were detected in the fractions collected during the enzyme purification, this using the glucose test kit (GOD-Perid method, Boehringer GmbH, Mannheim, Germany) in accordance with the instructions of the manufacturer.

Two 6-SFT isoforms with indistinguishable catalytic properties were isolated by the purification (table I). By affinity chromatography on the HighTrap blue column and by hydrophobic interaction chromatography on the alkylsuperose column the invertase (beta-fructosidase) activity was almost completely separated from the 6-SFT. This means that 6-SFT has no invertase activity. The mol ratio between beta-fructosidase and fructosyltransferase activity fell by a factor 6 after affinity chromatography and was then further reduced to a final ratio of approximately three after hydrophobic interaction chromatography (table I). The remaining beta-fructosidase activity could not be separated from 6-SFT and therefore appears to be one of its catalytic properties.

As already demonstrated by Simmen et. al., (supra), its capacity to transfer fructose to either sucrose or to iso-

kestose is a characterizing property of 6-SFT. Both 6-SFT isoforms which were obtained after the second anion exchange column have the same catalytic properties as shown by HPLC-analysis of the products formed after incubation with sucrose alone or with sucrose and isokestose (fig. 5B). In the presence of sucrose as the only substrate, mainly kestose is formed but sucrose is likewise hydrolysed to glucose and fructose. After incubation with sucrose and isokestose, mainly bifurcose is formed and much less sucrose is hydrolysed. This indicates that isokestose is the preferred acceptor compared with sucrose and that the beta-fructosidase activity is a component of the 6-SFT.

3. Gel electrophoresis

To illustrate the purity of the two 6-SFT isoforms fractions of the Resource Q chromatography lying between the two 6-SFT peaks, and therefore containing both fractions, were pooled (pool II in fig. 5) and analysed by non-denaturing IEF gel-analysis combined with either an enzyme activity assay (fig. 6A) or with SDS-PAGE analysis (fig. 6B).

For two-dimensional electrophoresis of 6-SFT pool II was subjected to isoelectric focussing within a pH range of 4-8 under non-denaturing conditions making use of a Mini-Protean II 2D-cell (Biorad) in accordance with the protocol of the manufacturer.

The 1 mm tubular gels were subsequently either cultured for 30 minutes in 5x sample buffer and loaded onto a 7.5-12% SDS polyacrylamide gel for a separation in the second dimension (Laemmli, Nature 227, 680-685, (1970)), or washed three times for ten minutes in 0.5 M citric acid Na_2HPO_4 buffer (pH 5.75) and cut into pieces of 2.5 mm for an enzyme activity assay. The 2.5 mm gel pieces were incubated in 0.4 M citric acid Na_2HPO_4 buffer (pH 5.75) with 0.2 M sucrose and 0.02% NaN_3 for 12 hours at 27°C. After centrifugation at 13,000 g for 5 minutes the supernatant was collected, heated to 95°C for 3 minutes, supplemented with trehalose (internal standard, final concentration 0.1 µg/µl) and stored at -20°C for further analysis.

Proteins separated on SDS-polyacrylamide gels were made visible by means of a silver staining (Blum, 1987).

The two isoforms were clearly separated and both had a fructosyltransferase and likewise a beta-fructosidase activity. Their pI differed only slightly and was close to pH 5.0. After denaturation both 6-SFT isoforms provided on SDS-PAGE two subunits of respectively 49 and 23 kDa. This data and the almost complete identicity of the fragment patterns obtained by tryptic digestion (data not shown) indicate that the two isoforms display very many similarities in respect of structure and sequence. The negatively loaded 6-SFT (containing both isoforms) had a molecular weight of approximately 67 kDa as determined by size-exclusion chromatography (data not shown).

4. Determination of the N-terminal amino acid sequence

For N-terminal amino acid sequence determination 100 µg protein of 6-SFT pool I and pool III was loaded onto a gradient gel (7.5-12%) and separated by SDS-PAGE (Laemmli, supra). The proteins were transferred to a polyvinylidene difluoride membrane (Immobilon PVDF transfer membrane, Millipore Corp., Bedford, MA) making use of the CAPS buffer system (Matsudeira, J. Biol. Chem 262, 10035-10038 (1987)). The protein bands were made visible on the membrane with 0.2% Ponceau S in 1% acetic acid, cut out and digested with trypsin.

Tryptic peptides were separated by reverse phase HPLC and N-terminal sequence determination of tryptic peptides was performed by automated Edman degradation.

The peptide sequence of the N-terminus of the 49 kDa subunit was determined and both, the large and the small, subunits were digested with trypsin in order to obtain internal peptide sequences. For both subunits two amino acid sequences of tryptic peptides were determined and used to design DNA primers (fig. 7).

5. Design of a probe

A 397 bp fragment was generated by reverse transcription polymerase chain reaction (RT-PCR). For this purpose single-strand cDNA was synthesized by reverse transcription of Poly(A^+)-RNA making use of a synthetic oligo-d(T) primer (23mer) and M-MuL V reverse transcriptase. PCR was performed according to the Perkin-Elmer protocol between the two synthetic, degenerated primers:

(i) CGCCTGCAGGTACCACATGTT(C/T)TA(C/T)CA(A/G)TA(C/T)AA(C/T)CC

(ii)CCACGTCTAGAGCTCTC(A/G)TC(A/G)TACCA(A/C/G)GC(C/G)GTCAT

These primers were designed in accordance with two part sequences of peptides obtained after tryptic digestion of 6-SFT. The resulting PCR product was cloned in the pCR-II™ vector (TA-cloning kit, Invitrogen). Labelling of the fragment with α-^{32}P-dATP was performed with a random primed labelling kit (Boehringer GmbH, Mannheim, Germany) according to the instructions of the manufacturer.

6. Screening of a cDNA library

The fragment of 397 bp generated as according to the method under 5. was used as a probe in an RNA gel blot analysis of primary leaves, in which the accumulation of fructans was induced by continuous exposure to light for different times. There was found to be no hybridisation signal in the case of untreated leaves while a hybridising band of approximately 1800 bp accumulated rapidly in a manner which corresponded with the increase in 6-SFT activity in the leaves (data not shown). This result points to the presence of a messenger RNA of about 1800 bp in length.

The PCR product was also used to screen a cDNA expression library of primary leaves. A search was made here for a cDNA of full length.

To this end a cDNA expression library was first manufactured by extracting total RNA from 8 day-old cut primary leaves in which the synthesis of fructans was induced by continuous exposure to light for 48 hours. The leaves were ground in liquid nitrogen to a fine powder and suspended in RNA extraction buffer (0.1 M Tris (HCl), pH 9, with 10mM

EDTA, 0.1 M NaCl and 25 mM DTT). The still frozen sample was further ground until a cream-like consistency was reached and the sample was then extracted with phenol-chloroform-isoamylalcohol (25:24:1;v:v:v) (Brandt and Ingversen, Carlsberg Res. Commum. 43, 451-469, 1978). The method was modified somewhat by omitting a second homogenisation step and by precipitating the RNA overnight with 2M LiCl at 4°C after the last chloroform extraction. After a final ethanol precipitation poly $(A)^+$-RNA was isolated by poly(U)-Sepharose chromatography (Brandt and Ingversen, supra) and used for cDNA synthesis (ZAP-cDNA synthesis Kit, Stratagene, LaJolla, Ca, USA).

The cDNA was ligated in a uni-ZAP-XR vector, digested with EcoRI and XhoI and packaged in phage coats (Gigapack III Packaging Kit, Stratagene, La Jolla, Ca, USA) (7.5×10^7 plaque-forming units per 5 µg poly$(A)^+$-RNA).

The primary library was screened with the α-^{32}P-labelled 397 bp long fragment of 6-SFT (see above) at 60°C in accordance with the Stratagene protocol. Positive clones were screened once again and Bluescript phagemides were finally cleaved from the resulting positive phages using the Exassist/SOLR-system (Strategene, La Jolla, Ca, USA). DNA sequencing of both strands was performed by the dideoxynucleotide sequencing method making use of the sequencing PRO kit (Toyobo, Osaka, Japan). Unless indicated otherwise, standard protocols were used (Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989)). Sequence data-analysis was carried out using the GCG sequence analyses software package, version 7.2 (1992).

After the first screening 9 positive clones were isolated. After a further screening 7 clones remained positive. Of these the sequence was partially determined from the 5' terminus and from the internal primers which were designed on the basis of the PCR product. All 7 clones appeared to code for the same protein, and four of them comprised the complete coding sequence. Of one of the possible clones of full length the sequence was wholly determined on both strands and it was found that it coded for a polypeptide

which contained the 49 kDa subunit as well as the 23 kDa subunit (fig. 7).

A schematic view of the complete nucleotide sequence of the fully sequenced cDNA is shown in fig. 8. It comprises one long open reading frame which begins at nucleotide 46 and ends at nucleotide 1923 for two stop codons. The open reading frame codes for a polypeptide chain of 626 amino acids including a leader sequence of 67 residues in length.

The mature 6-SFT starts at nucleotide 246 and therefore has at least 559 amino acid residues with a calculated molecular weight of 61.3 kDa and a calculated pI of 5.37. All 5 of the partial amino acid sequences obtained from the purified protein are present in the amino acid sequence derived from the cDNA (fig. 8). The cDNA likewise contains 45 bp of a 5' non-translated and 171 bp of a 3' non-translated sequence with a poly(A) tail. A possible translation initiation signal (ATG) of the 6-SFT cDNA is localized at the nucleotide positions 46 to 48 and a possible polyadenylating sequence is present at the nucleotide positions 1973 to 1979. It has been found that the mature 6-SFT displays alpha-methyl-mannoside-reversible binding on ConA-Sepharose, which indicates that it is a glycoprotein (data not shown). Similarly, the derived amino acid sequence contains 6 possible glycosylating positions (Asn-X-Ser/Thr).

All peptide sequences obtained from the purified protein are situated without any mismatch in the derived amino acid sequence. The two peptide sequences obtained from the 23 kDa subunit of the purified SFT are localized close to the 3'-terminus of the cDNA, while the sequences obtained from the 49 kDa subunit are localized in the vicinity of the 5'-terminus.

In order to study the possible relation of the cDNA to known beta-fructosidases and fructosyltransferases, the derived amino acid sequence was compared with the sequence of different vegetable, fungal and bacterial invertases, and with bacterial levanases and levansucrases (fig. 9 and fig. 10). The cDNA described herein has the highest homology with soluble acid invertases of the green soya bean (mungbean)

(Arai et al., supra), carrot (Unger et al., supra), and tomato (Elliott et al., supra), and equally clear homologies with invertases, levanases and levansucrases from other kingdoms, that is, with a number of beta-fructosidases. The comparison of the amino acid sequence indicates at least five well conserved domains. Domains I and IV are less conserved between invertases and levansucrases than domains II, III and V. With these enzymes domain III in particular is very conserved. Surprisingly, the most limited homology is that with bacterial levansucrases, that is, with a class of enzymes which catalyse a similar 6-fructosyl transfer reaction as 6-SFT (see the dendrogram in fig. 10).

7. Expression of 6-SFT in Nicotiana plumbaginifolia protoplasts

The 6-SFT cDNA clone was sub-cloned in a derivative of the pUC119 plasmid vector (Samsbrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring HArbor, 1989) under the regulation of the expression signals of the cauliflower mosaic virus 35S transcript (see Neuhaus et al., Proc. Natl. Acad. Sci. USA 88, 10362-10366 (1991)).

Protoplasts of Nicotiana plumbaginifolia were isolated and transformed largely as described by Goodall et al. (Meth. Enzymol. 181, 148-161 (1990)). In summary, 10 µg of the plasmid containing the 6-SFT cDNA was dispersed in a volume of 10 µl TE buffer in sterile 15 ml plastic tubes. Control transformations were carried out with 10 µg of the same plasmid without insert. 1 x 10^6 protoplasts were added up to a volume of 0.5 ml and mixed carefully with an equal volume 20% (w/v) polyethylene glycol 6000. After 2-5 minutes 6.5 ml K3 medium was added and the protoplasts incubated for two hours at 27°C. They were thereafter diluted 1:1 with the W5 osmoticum and pelleted for 10 minutes at 1000 g. All protoplasts (except those which were taken as control at t = 0 hour) were resuspended in 2 ml K3 medium and incubated at 27°C. After 3, 6, 9, 18 and 27 hours samples were taken for product analysis. The protoplasts were herein pelleted for

10 minutes at 1000 g after addition of 2 ml W5 osmoticum. The protoplast pellet was resuspended in 0.1 M citric acid Na_2HPO_4 buffer (pH 5.75), transferred to sterile Eppendorf tubes and frozen in liquid nitrogen. After defrosting the samples were vortexed, and cell debris was pelleted at 13.000 g for 3 minutes. The supernatants (50 to 100 µl) were desalted by guiding them over Biogel P-10 columns as described above. Desalted enzyme samples were incubated with 0.1 M sucrose or with 0.1 M sucrose in combination with 0.1 M isokestose in 50 mM citric acid Na_2HPO_4 buffer (pH 5.75) with 0.02% NaN_3 for 20 hours at 27°C. The product analysis was performed as described in the case of Fig. 5 after stopping of the reaction by heating the samples at 95°C for 3 minutes.

After an initial lag-phase of about 3 hours extracts of protoplasts formed kestose from sucrose and bifurcose from sucrose and isokestose. This confirms that the cDNA codes for a functional 6-SFT (fig. 10). Like the purified enzyme, the activity present in the protoplasts catalysed the production of bifurcose from sucrose and isokestose at a speed that was roughly four times higher than the production of isokestose from sucrose. These results confirm that the cDNA codes for a 6-SFT.

EXAMPLE 6

Fructan-fructan fructosyltransferase from Jerusalem artichoke

Another vegetable fructosyltransferase for application in the invention was purified from Jerusalem artichoke (Helianthus tuberosus L.) by means of the Lüscher method (Lüscher M. et al., New Phytol. 123, 717-724 (1993)) using salt precipitation, lectin-affinity chromatography and ion exchange chromatography.

The purified enzyme was separated on a native IEF-gel and blotted on a PVDF membrane. The membrane was stained by means of a Coomassie Blue staining and the two most impor-

tant FFT isoforms (respectively T1 and T2) were cut out (see figure 12).

Both proteins T1 and T2 were digested with trypsin and the peptides were separated by means of HPLC. The HPLC-diagrams of the digested FFT isoforms exhibit identical patterns (see figures 13 and 14). The amino acid sequence was determined of two of the purified peptides of T2 (fractions 18 and 24). The sequence of the first peptide was: NH_2 - E - Q - W - E - G - X - F - M - Q - Q - Y - X - X - The other peptide had the following amino acid sequence: NH_2 - A - V - P - V - X - L - X - X - P - L - (F/L) - I - X - W - V -.

In the same manner as in example 5 the cDNA was isolated and the sequence determined. Using a complete cDNA-clone plant cells were transformed to obtain transgenic plants.

EXAMPLE 7

Use of the oligosaccharides according to the invention

The oligosaccharides produced using the method according to the invention can be used as sugar substitutes in different products. Three examples hereof are given below.

1. Ice cream

Ice cream is prepared from the following ingredients:

635 parts water
90 parts butter fat
100 parts low-fat milk powder
170 parts oligosaccharides according to the invention
5 parts Cremodan SE30™ (Grindsted)
0.3 parts Aspartame™
flavourings as required.

The milk powder is dissolved in the water. The whole is heated to 40-45°C. The remaining dry ingredients are mixed and dissolved in the warm milk. The melted butter is then added. This whole is then pasteurised for 10 minutes at

72°C. The mixture is thereafter homogenised in a two-stage homogenizer at 150/35 bar. The thus obtained ice mix is cooled rapidly to 5°C and the whole is subsequently left to mature for a minimum of 4 hours at 5°C. Finally, the ice mix is aerated and frozen to an overrun of 100%.

After hardening at -35°C and storage at -20°C an ice cream is obtained which corresponds in terms of taste and texture with ice cream prepared with natural sugars (saccharose, glucose syrup).

2. Muesli bar

A muesli bar was prepared from the following ingredients:

28 parts oligosaccharides according to the invention
68 parts muesli mix
4 parts cacao

A syrup was produced from the oligosaccharides by heating, which syrup was mixed with the other ingredients. The bars were formed from the thus obtained mixture in a cylindrical press. Due to the omission of natural sugar the bar is much lower-calory than the conventional bars.

3. Soft drink

A soft drink was prepared from the following ingredients:

90 parts water or fruit juice
8-10 parts oligosaccharides according to the invention
artificial sweeteners
flavourings and coloring agents
nutrient acid
carbon dioxide

All ingredients were dissolved in a part of the water. The remaining water was then added as carbon dioxide-containing water. The energy value of the soft drink is much less because no additional natural sugars are added.

Table I
Purification of 6-SFT

Purification step	fructosyltransferase[a]		Protein	Purification	ß-fructosidase/-fructosyltransferase[b]
	nkatal[c]	%	mg	-fold	mol ratio
Crude extract	243	100	5000	1	32
Acid precipitation	159	66	1700	2	29
High-Trap-blue	71.6	29	450	3	5.7
First Resource Q	22.6	9.3	79	6	6.2
Alcyl Superose	9.32	3.8	56	4	3.2
Superdex 75	6.64	2.7	9.5	15	3.4
Second Resource Q pool I	2.99	1.2	0.6	103	2.7
Second Resource Q pool II	4.33	1.8	1.7	52	3.2

[a] measured as kestose-producing activity
[b] mol fructose per mol produced fructose
[c] nkatal = $nmol \cdot s^{-1}$